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## Immunostaining for the tumour suppressor gene p16 product is a useful marker to differentiate melanoma metastasis from lymph-node nevus

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**Abstract** Upon the introduction of extensive sampling protocols of sentinel node biopsies, pathologists are increasingly confronted with small melanoma metastases. Using conventional histology, it proves sometimes difficult or impossible to differentiate small melanoma metastases from lymph-node nevi. Loss of the tumour suppressor gene p16 has been shown to be associated with tumour progression of melanoma. We investigated nevus and melanoma cells for the presence of the product of the gene p16, using immunohistochemistry. All nevus cells, independent of their location (nodal or skin) displayed an extensive nuclear and cytoplasmic staining for p16. In contrast, all cells of melanoma metastases, except one skin metastasis, lacked nuclear staining for p16. These findings indicate that p16 is a reliable marker to distinguish lymph-node nevi from melanoma metastasis.

**Keywords** Nodal nevus · Metastatic melanoma · Sentinel lymph node · Tumour suppressor gene · p16

### Introduction

The reliable differentiation between lymph-node nevus and melanoma metastasis is sometimes difficult. The recommended marker panel for diagnosis of melanoma [S-100 protein, gp100 (HMB45), Melan-A/MART-1 (A103) and Tyrosinase (T311)] is not always useful to reliably discriminate the two lesions [4]. However, this differential diagnosis bears a particular importance in

sentinel lymph nodes, as the diagnosis of melanoma metastasis leads to therapeutic lymph-node dissection.

The product of the p16/INK4a/CDKN2/MTS tumour suppressor gene acts as a negative cell cycle regulator by inhibiting G1 cyclin-dependent kinases, which are phosphorylating the retinoblastoma protein [10, 18]. P16 is inactivated or lost in a wide range of human malignancies [15], including familial melanoma [3]. In sporadic melanoma, the results are still not conclusive [7, 8]. Loss or inactivation of the p16 gene has been shown to be associated with the progression of melanoma and other tumours [9, 16].

The goal of this study was to test the p16 as a marker to discriminate nodal nevi from melanoma metastases. We investigated dermal and nodal nevi, melanoma metastases of lymph nodes and other organs for loss of p16 expression, using immunohistochemistry.

### Materials and methods

#### Tissue samples

Formaldehyde-fixed and paraffin-embedded tissue specimens from the archives of the Department of Pathology, University of Zürich and of the Institute of Pathology, Kantonsspital Aarau (one sample) were investigated. Tested for the presence of p16 product were 18 nodal nevi of 13 patients, 26 sentinel lymph-node metastases of 19 melanoma patients, 10 non-sentinel lymph-node melanoma metastases of 10 patients and 13 organ metastases of melanoma of 12 patients. As lymph-node nevi are relatively uncommon [1, 17], and in this study most of them were from melanoma patients, we additionally tested 15 skin nevi (9 dermal, 5 compound and 1 junctional) of 9 patients. Haematoxylin and eosin-stained sections of all samples were reviewed by one pathologist (D.M.). For clinical data, see Table 1, Table 2 and Table 3.

#### Immunohistochemistry

A monoclonal primary antibody directed against an epitope of p16 (Neomarkers, Fremont, CA, USA) was used. The epitope of Ab-4 maps among amino acids 1–32 of the p16/INK4a protein.

Two-micron-thick sections of paraffin-embedded tissue were collected onto Superfrost Plus slides (Menzel Gläser, Germany) and dewaxed in graded alcohols. Non-specific peroxidase activity was

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**Table 1** Lymph-node nevi. *Nuc* nuclear staining, *CS* cytoplasmic without nuclear staining, *NP* no proliferation

Patient no.	Age (years)/sex	Site of node	Primary tumor	Operation	Size (mm)	S-100 P	HMB 45	P16 Nuc	P16 CS	MIB-1
1	49/Female	Axilla	Melanoma	Node dissection	7	4+	0	4+	4+	NP
2	67/Male	Neck	Melanoma	Node dissection	0.5	4+	0	4+	4+	*
3	30/Female	Axilla	Melanoma	Sentinel node	0.4	4+	0	3+	3+	NP
4	57/Female	Inguina	Melanoma	Sentinel node	0.5	4+	0	4+	4+	NP
5	66/Male	Axilla	Melanoma	Sentinel node	2.5	4+	0	3+	3+	<0.2%
6a	61/Male	Inguina	Melanoma	Sentinel node	5	4+	0	3+	3+	<0.3%
b		Inguina		Sentinel node	3	4+	0	4+	4+	<0.3%
c		Inguina		Node dissection	0.5	4+	0	3+	3+	NP
d		Inguina		Node dissection	2	4+	0	3+	3+	<0.3%
7a	63/Male	Axilla	Melanoma	Sentinel node	0.5	4+	0	4+	4+	NP
b		Axilla		Sentinel node	0.3	4+	0	4+	4+	NP
8	67/Female	Inguina	No	Femoral hernia	0.8	4+	0	3+	3+	<0.2%
9a	66/Female	Axilla	Breast cancer	Node dissection	20	4+	0	4+	4+	NP
b		Axilla		Node dissection	2.5	4+	0	4+	4+	NP
10	20/Male	Inguina	Melanoma	Sentinel node	0.1	4+	0	4+	4+	NP
11	50/Female	Axilla	Melanoma	Sentinel node	2.5	4+	0	4+	4+	NP
12	61/Male	Axilla	Melanoma	Sentinel node	0.2	4+	0	4+	4+	NP
13	60/Male	Neck	Melanoma	Sentinel node	0.2	4+	0	4+	4+	NP

\* On additional serial sections the lesion was no longer present

**Table 2** Sentinel lymph-node metastases. *Nuc* nuclear staining, *CS* cytoplasmic without nuclear staining, *NP* no proliferation

Patient no.	Age (years)/sex	Site of node	Size (mm)	S-100 P	HMB 45	P16 Nuc	P16 CS	MIB-1
1	57/Male	Axilla	2.5	4+	0	0	0	*
2	50/Male	Infraclavicular	0.5	4+	4+	0	0	*
3	62/Male	Ocipital	1	4+	4+	0	0	25–50%
4	68/Male	Axilla	0.7	4+	0	0	0	10–25%
5a	64/Male	Axilla	1.4	4+	1+	0	3+	10–25%
b		Axilla	1.3	4+	2+	0	2+	10–25%
6	61/Male	Ocipital	2.5	4+	2+	0	2+	6%
7	48/Female	Axilla	1	4+	4+	0	0	25–50%
8	59/Male	Inguina	8	4+	4+	0	3+	25–50%
9	58/Male	Inguina	0.6	4+	2+	0	0	10–25%
10a	68/Male	Inguina	0.2	4+	4+	0	0	4%
b		Inguina	1	4+	4+	0	3+	25–50%
11a	64/Male	Elbow	1	4+	0	0	0	10–25%
b		Axilla	0.6	4+	1+	0	0	10–25%
12a	74/Female	Inguina	0.8	4+	1+	0	0	10–25%
b		Inguina	0.4	4+	0	0	0	3%
13	63/Female	Inguina	2	4+	4+	0	0	10–25%
14	60/Male	Inguina	1	4+	0	0	0	NP
15	22/Female	Inguina	1.2	4+	2+	0	1+	*
16	59/Male	Axilla	3.5	4+	3+	0	0	10–25%
17a	19/Female	Ocipital	0.5	4+	0	0	0	4%
b		Ocipital	0.8	4+	0	0	0	5%
18	61/Male	Inguina	0.7	4+	0	0	0	10–25%
19a	71/Male	Axilla	0.7	4+	1+	0	0	10–25%
b		Axilla	0.2	4+	1+	0	0	10–25%
c		Axilla	0.1	4+	1+	0	0	10–25%

\* On additional serial sections the lesion was no longer present

blocked with 3% hydrogen peroxide in phosphate buffer saline for 10 min. Microwave pre-treatment was performed for 10 min in 0.01 mol/l tri-sodium citrate puffer at pH 6.3. The sections were incubated with p16 antibody (Clone 16P04; Neomarkers, Fremont, CA, USA) at an antibody concentration of 40 µg/ml at room temperature for 2 h. P16 detection was performed with “Elite” universal detection kit (Vector, Peterborough, UK) and colour was developed using diaminobenzidine. A dermal nevus, an oligoastrocytoma (G2, World Health Organization) and endocrine cells of the pancreas were used as positive controls. Negative controls were performed by replacing the specific antiserum with pure Tris saline.

We differentiated nuclear and cytoplasmic staining from exclusive cytoplasmic staining. The extent of each staining was recorded according to the following system: 0=no staining; 1+=<25% of cells positive; 2+=25–49% of cells positive; 3+=50–74% of cells positive; 4+=>75% cells positive.

For Ki-67, HMB45 and S-100 protein, immunohistochemistry was performed on paraffin sections of formalin-fixed tissues, using a Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, Arizona). For antigen retrieval, slides were heated with cell conditioner 1 (mild protocol). Primary antibodies (all from DakoCytomation, Glostrup, Denmark) against the following antigens were applied: Ki-67 proliferation antigen (clone

**Table 3** Melanoma metastases: node dissection and organ metastasis. *Nuc* nuclear staining, *CS* cytoplasmic without nuclear staining

Patient no.	Age (years)/sex	Site	S-100 P	HMB 45	P 16 Nuc	P16 CS	MIB-1
1	77/Male	Neck*	4+	0	0	4+	10–25%
2	54/Female	Infraclavicular*	4+	2+	0	0	5%
3	78/Male	Inguinal*	4+	1+	0	0	25–50%
4	40/Male	Neck*	4+	0	0	4+	25–50%
5	59/Female	Neck*	4+	4+	0	0	25–50%
6	75/Male	Axilla*	4+	3+	0	0	10–25%
7	47/Female	Axilla*	4+	3+	0	0	8%
8	73/Male	Axilla*	4+	0	0	3+	25–50%
9	36/Male	Axilla*	4+	1+	0	0	25–50%
10	56/Female	Retroauricular*	4+	2+	0	0	10–25%
11	30/Female	Liver	4+	2+	0	1+	10–25%
12	67/Male	Skin (thorax)	3+	3+	0	0	10–25%
13	47/Male	Subcutis (face)	4+	0	0	0	10–25%
14	59/Male	Small bowel	4+	1+	0	4+	10–25%
15	69/Male	Skin (inguina)	4+	4+	1+	3+	25–50%
16	60/Male	Parotid gland	4+	0	0	0	10–25%
17	59/Male	Soft tissue axilla	4+	1+	0	0	25–50%
18	58/Male	Soft tissue axilla	4+	1+	0	0	10–25%
		Soft tissue axilla	4+	2+	0	0	10–25%
19	56/Male	Skin (thorax)	4+	2+	0	4+	25–50%
20	69/Male	Subcutis (inguina)	4+	4+	0	0	4%
21	47/Female	Soft tissue axilla	4+	4+	0	0	10–25%
22	63/Male	Skin	4+	0	0	0	25–50%

\* Node dissection

MIB-1, dilution 1:20), melanosome antigen (clone HMB45, dilution 1:50) and S-100 protein (rabbit affinity purified antibodies, dilution 1:1000). Monoclonal mouse MIB-1 antibodies were detected with the Ventana iVIEW DAB detection kit, yielding a brown reaction product. Monoclonal mouse HMB45 and polyclonal rabbit anti-S-100 antibodies were revealed with the Ventana-Red enhanced detection kit (red reaction product). For HMB45, the signal was enhanced with the Ventana amplification kit. Slides were counterstained with haematoxylin. MIB-1 labelling of lymph-node nevi and melanoma metastases was recorded as the ratio of marked nuclei (cells) of the total number of cells per visual field.

## Results

### Clinical findings

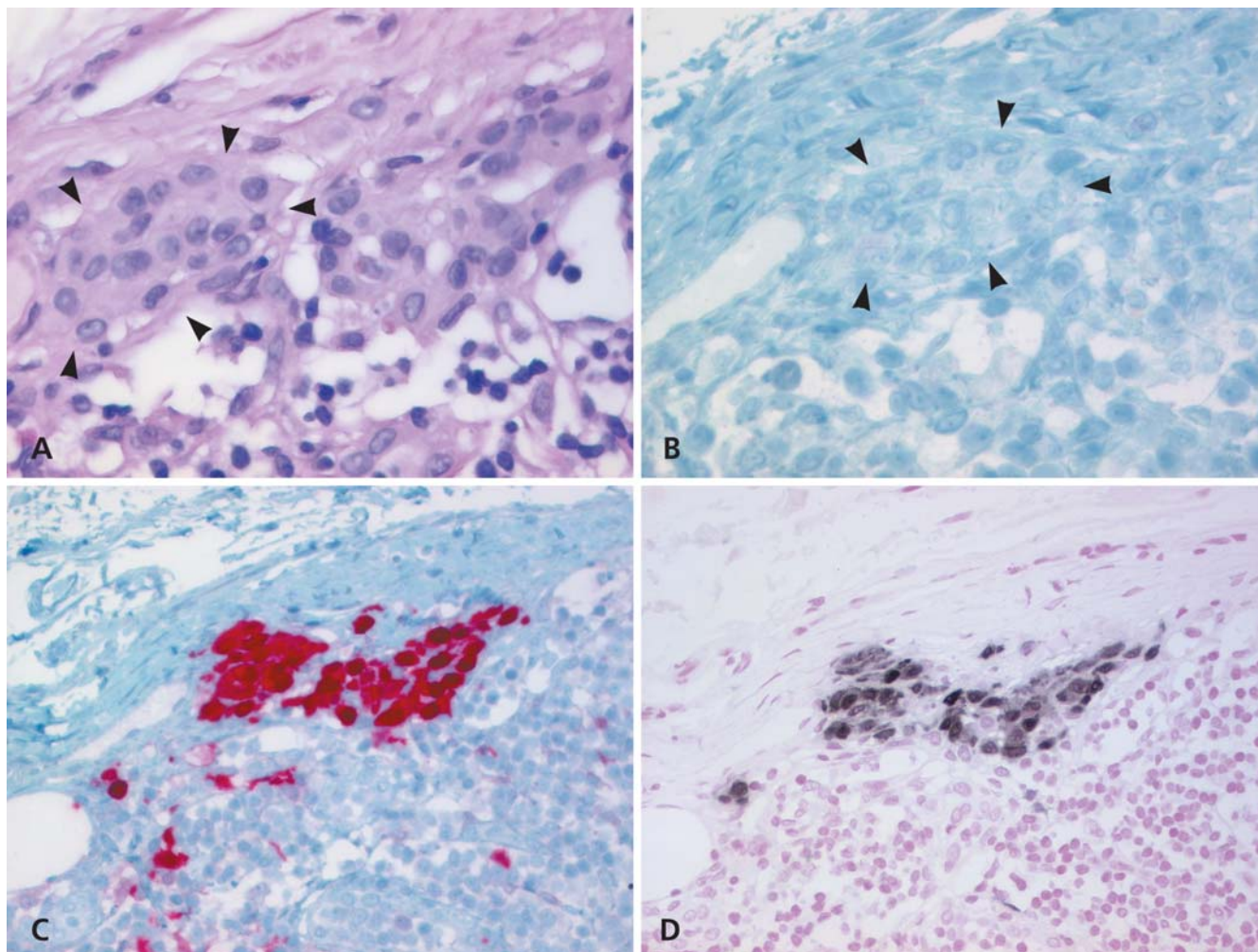
The clinical findings in 13 patients (20–67 years old) with nodal nevi, 22 patients (30–78 years old) with melanoma metastasis and 19 patients (19–74 years old) with sentinel node melanoma metastasis are listed in Table 1, Table 2 and Table 3. Nine nodal nevi were located in axillary lymph nodes, seven in inguinal nodes and two in neck nodes. Of the patients, 11 had undergone lymph-node biopsies for cutaneous melanoma, 1 patient for breast cancer and 1 for a femoral hernia. In all but one (patient 6) patient with nodal nevi, neither concomitant melanoma metastasis nor clinical signs of metastasis were present (mean clinical follow-up to 2 years). In nine patients (6–68 years old), 9 of 15 skin nevi were dermal, 5 were compound and 1 was junctional.

### Histopathology

For a lesion to be accepted as lymph-node nevus, aggregates of cytologically typical melanocytes must be present in the lymph-node capsule and/or fibrous trabeculae. Only three large nodal nevi (patients 6a, b, d) and one small nodal nevus (0.4 mm, patient 3, Fig. 1) did not fulfil these conditions, as they were partially localised within the lymph node and were in direct contact with its parenchyma. The benign nature of these four lesions was confirmed by the presence of cytologically typical melanocytes, a very low (<0.3%) proliferation index in combination with a clear-cut nuclear staining for p16. Patient 6 suffered from an additional sentinel node micrometastasis (registered as patient 18) displaying a different, malignant (atypical) morphology of the melanocytes, a distinctly higher proliferation index (18%) and an absent staining for p16.

The histological findings are illustrated in Fig. 1, Fig. 2, Fig. 3 and Fig. 4 and listed in Table 1, Table 2 and Table 3. The size of nodal nevi (measured as the single greatest dimension) ranged from 0.1 mm to 20 mm with an average of 2.7 mm. Of 18 nodal nevi, 11 were small (maximum 2 mm). The size of melanoma metastasis in sentinel lymph nodes ranged from 0.1 mm to 8 mm with an average of 1.3 mm. The diameter of 21 of 26 sentinel node metastases measured less than 2 mm. Diagnostic difficulties occurred in two lymph-node metastases, as they were partially localised within the capsule and were almost lacking HMB 45 reactivity (patients 5a and 12b). Melanoma micrometastasis in the lymph-node parenchyma displaying an identical morphology and immunohistochemistry confirmed the metastatic origin of these cells (Fig. 2).





**Fig. 1** Nodal nevus (patient 3). **A** Cluster of melanocytes lacking clear-cut atypia but extending into the lymph node parenchyma (haematoxylin-eosin,  $\times 500$ ). **B** Lack of immunostaining for HMB

45 ( $\times 500$ ). **C** Immunostaining for S-100 protein ( $\times 250$ ). **D** 3+ nuclear and cytoplasmic immunostaining for p16 ( $\times 250$ )

### Immunohistochemistry

We found two types of immunoreactions of p16 in melanocytes: nuclear and cytoplasmic staining and diffuse cytoplasmic staining with absence of nuclear staining. All 33 nevi (18 nodal and 15 dermal) displayed an extensive and intense nuclear and cytoplasmic staining, whereas nuclear staining was absent in 48 of 49 melanoma metastases (26 sentinel nodes, 10 non-sentinel nodes and 13 organ metastases) (Fig. 3). Only one inguinal skin metastasis displayed a focal weak (1+) nuclear positivity. In contrast, melanoma metastases in 6 of 26 sentinel nodes, in 3 of 10 non-sentinel nodes and in 4 of 13 organ metastases displayed an exclusive cytoplasmic staining (Fig. 3).

### Controls

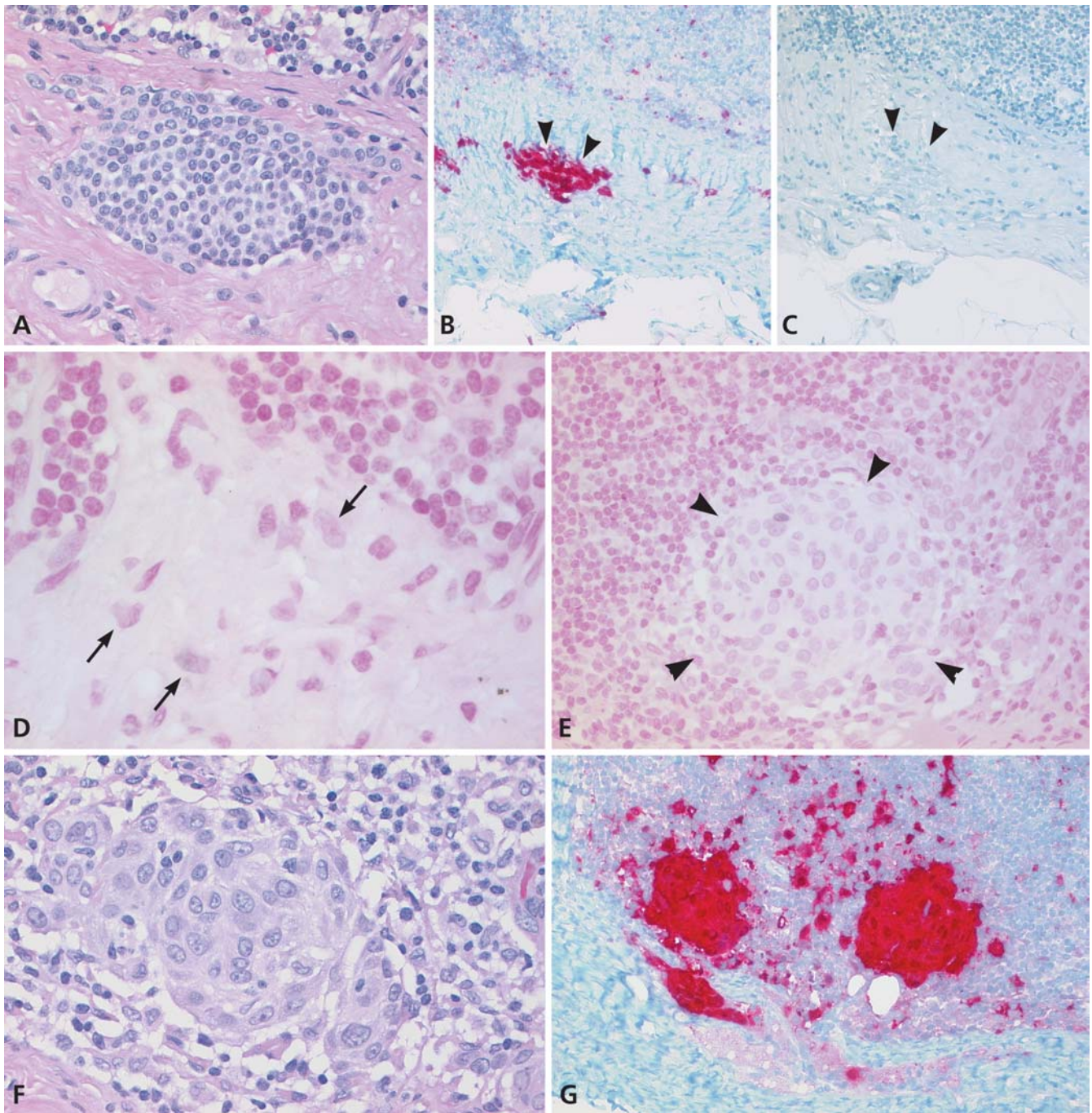
All positive controls displayed an extensive and intense 4+ positive combined nuclear and cytoplasmic staining.

All negative controls lacked cells displaying an immunoreaction. Except for one sentinel node metastasis, all melanoma metastases displayed an elevated proliferative index as shown by MIB-1 staining. Of melanoma metastases, 8 showed an index in less than 10% of melanoma cells; the index was 10–25% in 24 melanoma metastases, and 25–50% in 13 melanoma metastases. Of lymph-node nevi, 12 showed no proliferation while 5 showed a proliferative index of less than 0.3%. Three sentinel-node metastases and one lymph-node nevus could not be stained with Ki-67 because the lesion was not present in additional serial sections. The detailed results of immunohistochemical staining for p16 and Ki-67 are shown in Table 1, Table 2 and Table 3.

### Discussion

The differential diagnosis of lymph-node nevus and melanoma metastasis becomes increasingly important in the investigation of sentinel lymph nodes. It is, however,





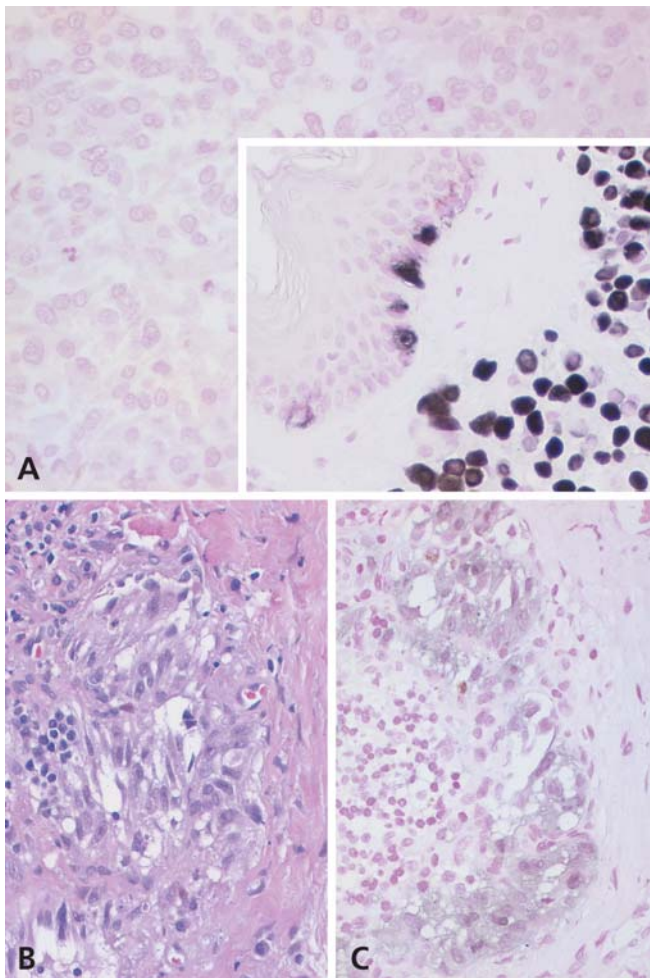
**Fig. 2** Melanoma metastasis in a sentinel-node biopsy (patient, 12b). **A** A small number of melanoma cells are confined to the lymphatic space within the capsule and are not markedly atypical (haematoxylin-eosin,  $\times 300$ ). **B** S-100 protein immunostaining ( $\times 125$ ). The melanoma cells are negative for HMB 45 ( $\times 125$ ; **C**)

and p16 ( $\times 500$ ; **D**). **E** A micrometastasis with identical morphology in the parenchyma of the same lymph node confirms the diagnosis of the presence of melanoma cells within the capsule. Lack of immunostaining for p16 ( $\times 250$ ). **F** Haematoxylin-eosin ( $\times 320$ ). **G** Immunostaining for S-100 protein ( $\times 150$ )

difficult to reliably distinguish lymph-node nevi from metastases of melanoma using conventional histology. This difficulty is partly due to the nevoid pattern of some melanoma metastases, which may be devoid of clear-cut atypical cells (Fig. 2) [5, 11, 12, 19, 21]. Even upon comparing the primary with its metastasi(e)s, it is sometimes less than obvious that the lymph-node lesion represents a metastasis. The localisation within the

lymph-node capsule is an important diagnostic criterion for the diagnosis of nodal nevi [2, 14]. However, this criterion proves not to be entirely reliable, as melanoma metastases are sometimes confined to the lymphatic space adjacent to the lymph-node capsule (Fig. 2) and nodal nevi may show parenchymal involvement [20]. The demonstration of the expression of HMB 45 may be helpful, although not entirely reliable, as a loss of HMB



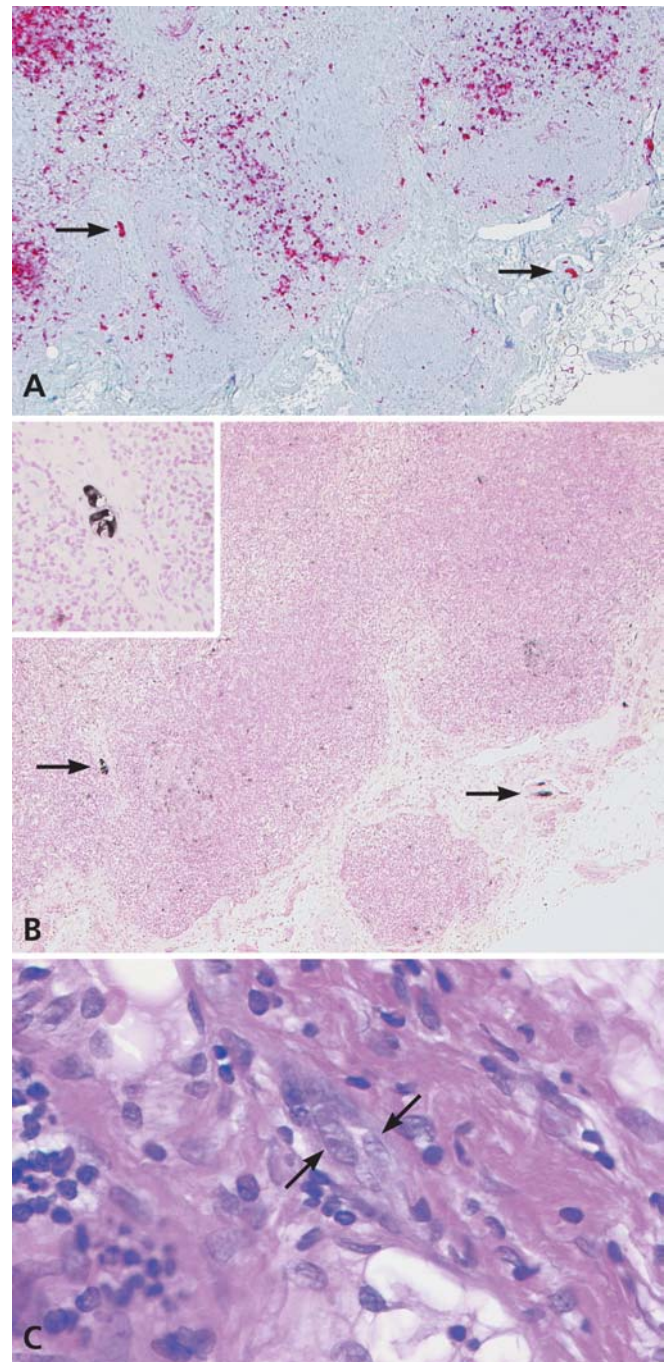


**Fig. 3** Soft tissue metastasis of the axilla (patient 17, **A**) and inguinal sentinel lymph-node metastasis (patient 8, **B** and **C**). **A** Lack of immunostaining for p16 ( $\times 250$ ). Dermal nevus with 4+ nuclear and cytoplasmic immunostaining for p16 as positive control (*inset*,  $\times 200$ ). **B** Haematoxylin-eosin ( $\times 200$ ). **C** Weak cytoplasmic staining in absence of nuclear immunostaining for p16 ( $\times 200$ )

45 expression is reported in at least 20% of melanoma metastases [4].

We demonstrated a clear-cut difference in the staining pattern of p16 between lymph-node nevi and melanoma metastases. Using immunohistochemistry, we found nuclear and cytoplasmic p16 staining in all nevi, independent of their localisation (dermal or lymph node). Furthermore, we have demonstrated the absence of nuclear staining in 48 melanoma metastases, except for 1 skin metastasis, which displayed a weak nuclear immunoreaction. However, a diffuse cytoplasmic staining in the absence of nuclear staining was present in 13 of 49 melanoma metastases.

P16 immunohistochemistry often revealed a larger extent of nodal nevi in comparison with tinctorial staining. Sometimes small melanocyte groups are present within the lymph-node capsule. They may, therefore, be difficult to recognise, even upon using S-100 protein immunohistochemistry, because—depending on the anti-



**Fig. 4** Histology of a small cluster of intracapsular melanocytes. **A** Melanocytes are masked by S-100 protein-immunostained dendritic cells ( $\times 35$ ). **B** Some intracapsular melanocytes detected with 4+ nuclear and cytoplasmic immunostaining for p16,  $\times 35$  (*inset*,  $\times 250$ ). **C** Haematoxylin-eosin ( $\times 400$ )

body used—they may be masked by dendritic cells (Fig. 4). Nodal nevi were reported to occur from less than 1% of axillary nodes dissected for mammary carcinoma [1, 17] up to 3.9% in sentinel lymph nodes of various localisations of patients suffering from cutaneous melanoma [2]. Due to the aforementioned diagnostic difficul-

**Table 4** Immunohistochemical findings in lymph-node nevi and melanoma metastases. + positive, -negative, (+) rarely positive, (-) rarely negative, ((+)) very rarely positive, ((-)) very rarely

	P16 Nuc	P16 CS	HMB45	Melan A	Tyrosinasee	S-100 P	MIB-1
Lymph-node nevi	+	+	-/(+)	+/-	+/-	+	NP or <0.3%
Melanoma metastases	-/(+)	-/(+)	+/-	+/-	+/-	+/(--)	10% or more*

\* Rarely no proliferation or less than 10%

ties, the real incidence of nodal nevi might be higher than hitherto reported.

In some larger nevi, we found p16-positive cells displaying co-expression of S-100 protein in the lymph-node parenchyma. We, therefore, assume these cells to be melanocytes. This finding adds support to the hypothesis of the embolic origin of lymph-node nevi [2], which was put forward in previous reports in which melanocytes have been demonstrated to occur in the sinus of lymph nodes [20]. A recent study [6] has noted a strong association between nodal and cutaneous nevi.

A recent report showed that lack of MIB-1 staining or a labelling index below 1% strongly favours nevus versus melanoma metastasis [13]. This is consistent with our assessment of the proliferation index in nevi and melanoma metastases. Unfortunately it may prove difficult or impossible to precisely define a labelling index in very small lesions.

We consider immunostaining for p16 an important additional marker to differentiate nodal nevi from melanoma metastases and, therefore, recommend its use (Table 4).

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